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Optimized Preparation of Levofloxacin-loaded Chitosan Nanoparticles by Ionotropic Gelation

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Abstract

The present work investigates the feasibility of fabricating chitosan (CS)-levofloxacin (LOF) nanoparticles by ionotropic gelation technology. An orthogonal experiment was designed to optimize its preparing parameters and multi-index comprehensive weighed score analysis method was used to study the effects of various factors including concentration of CS, concentration of tripolyphosphate (TPP), mass ratio of CS to TPP, and mass ratio of CS to LOF on the properties of nanoparticles. The particles prepared under optimal condition of 2mg/ml CS concentration, 2mg/ml TPP concentration, 0.5:1 mass ratio of oil to water and 4:1 mass ratio of CS to TPP had 140nm diameter, 0.95 span, 6.13% loading capacity (LC) and 24.91% encapsulation efficiency (EE). *In vitro* release profile showed that LOF released fast initially and then slowly with T_{90} occurring at 76.5h. Future studies should focus on antibacterial and biocompatible properties in order to evaluate its potential as sustainable delivery system.

Keywords: levofloxacin; chitosan; ionotropic gelation; nanoparticle; preparation

1. Introduction

Levofloxacin (LOF) is one of the third generations of fluoroquinolones with broad-spectrum antibacterial activity. It is effective to either gram positive or gram negative bacteria, thus is widely used for controlling urinary tract, respiratory tract, skin and soft tissue infections. However, it requires frequent dosing to maintain therapeutic effect because of its short biological half-life and greatly varying pharmaceutical concentrations of blood. The normal dosage regimen of this drug is 100 mg, usually administered three times a day [1]. But in some severe cases, long-term therapy might also be required. Sustained delivery LOF preparation can be helpful to conserve and maintain effective drug concentration, reduce dosing times, improve compliance and decrease side effects, and thus, optimize drug therapy.

Many efforts towards developing LOF sustained delivery system in the form of capsulate [2], filament [3], microsphere [4-6], and nanoparticle [7,8] have been made during the past few years. Since nanoparticle has decreased particle size, increased surface area, enhanced reactivity, promoted drug dissolution, reformed targeting, reduced toxicity and improved sustained-release efficacy, and therefore it could offer numerous advantages over the conventional dosage forms and attracted a considerable attention recently [9].

Compared with other carriers, chitosan (CS) has many advantages for controlling drugs delivery, particularly for microsphere or nanoparticle delivery system. These include its ability to control the release of active agents, avoiding the

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use of hazardous organic solvents while fabricating particles since it is soluble in aqueous acidic solution, being a linear polyamine containing a number of free amine groups that are readily available for crosslinking, and so on [10-15].

Among various CS nanoparticle preparation methods, ionotropic gelatin is a simpler, milder and safer one since CS nanoparticles are formed by electrostatic interaction, instead of chemical crosslinking. Tripolyphosphate (TPP) is such a polyanion that can interact with the cationic CS by electrostatic forces. In this method, CS is dissolved in aqueous acidic solution to obtain the cations of CS. This solution is then added dropwise under constant stirring into TPP solution. Due to the complex between oppositely charged species, CS undergoes ionic gelation and precipitates to form spherical particles [16].

After Bodmeier reported the preparation of CS-TPP complex by dropping CS droplets into a TPP solution [17], many researchers have explored its potential pharmaceutical usage, especially for nanoparticle sustainable release system. Previous studies showed that the physicochemical properties, stability and drug release property of those nanoparticles were mostly affected by the molecular weight (MW), deacetylation degree (DD) and concentration of CS, concentration of TPP, the ratio of CS to TPP, and drug content [18-20]. However, those particles have never been tried on loading LOF until now.

In this paper, CS with 98000 of MW and ≥87% of DD was chosen as the carrier of LOF. An orthogonal test was designed to optimize ionic gelatin preparing parameters for nanoparticles, and the effects of various factors, including concentration of CS, concentration of TPP, mass ratio of CS to TPP, and mass ratio of CS to LOF, on the comprehensive properties of nanoparticles, involving particle size, span, loading capacity (LC) and encapsulation efficiency (EE) were evaluated based on the multi-index comprehensive weighed score analysis method. Then, the physical-chemical and drug release property were tested to verify its potential as sustainable delivery system.

2. Materials and Methods

2.1. Materials

LOF (batch number: 200801012, Zhejiang East Pharmaceutical Co., China); CS (MW: 98000; DD> 87%, Shandong Aokang Biological Technology Co., China); TPP（Sigma, USA）; glacial acetic acid, hydrochloric acid (Tianjin Chemical Reagent Co., China).

2.2. Preparation of nanoparticles

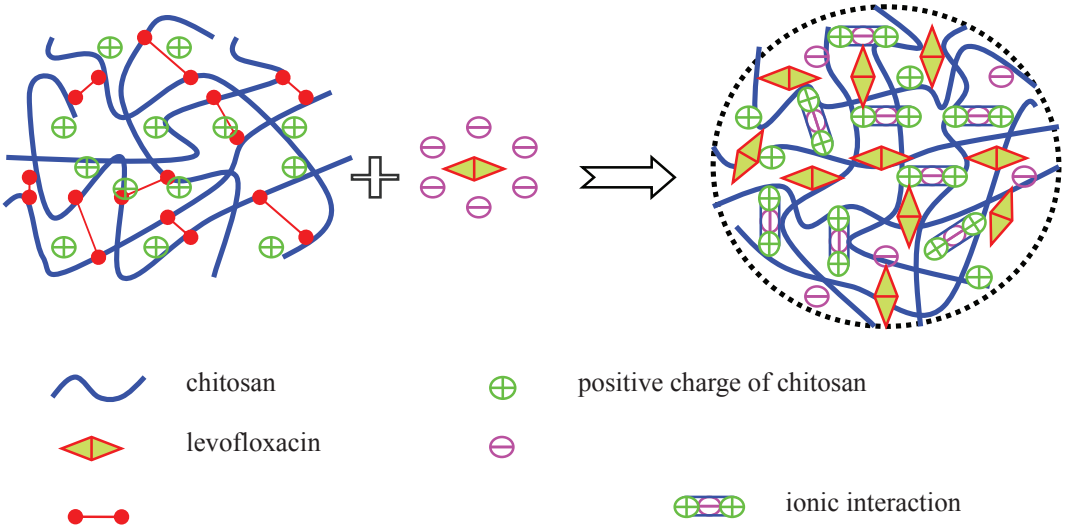


Fig.1. Schematic drawing of CS-TPP nanoparticle formation by ionotropic gelation

Suitable amount of LOF was put into 20ml of 1 ~ 2 mg/ml CS acetic solution under 500 rpm stirring rate until dissolution. This solution was then added dropwise into certain amount of 1 ~ 2 mg/ml TPP solution, and pH was adjusted

into the range from 4.5 to 6. Then nanoparticle suspensions can be formed and its mechanism of formation was demonstrated by Fig.1.

2.3. Morphological detection

The nanoparticle suspension was drawn into a piece of glass slide and observed under biological microscopy with 1000 magnification. For TEM, the nanoparticle solution was dropped on copper grids and dried overnight at room temperature for viewing (Philips EM400ST).

2.4. Size of nanoparticles

Nanoparticles were diluted by distilled water to form dispersion. The sizes of those nanoparticles in fluid were measured by laser particle size analyzer at 25℃. The span was calculated by the following formula:

$$Span=（D_{90}-D_{10}）/D_{50} \quad (1)$$

Where D_{90}、D_{50} and D_{10} designates that the particle size for which 90%、50% and 10% of the particles are smaller than this volume respectively.

2.5. Standard curve of LOF

About 5mg LOF was weighed accurately and placed into 50 ml volumetric flask. Then PBS solution with pH 7.4 was added into the flask to make 0.1mg/mL LOF stock solution. Then the stock solution was diluted into series of standard solutions with LOF concentrations of 3 μ g/ml, 6 μ g/ml, 9 μ g/ml, 12 μ g/ml and 15 μ g/ml respectively. The absorbance of each solution at 293nm was determined by UV spectrophotometer with PBS as blank. Then LOF standard curve was drawn and the corresponding data was regressed as standard equation.

2.6. LC and EE of nanoparticles

The LC and EE of nanoparticles were determined by separation of nanoparticles from the aqueous medium containing free drug by centrifugation at 14000rpm for 30 min. The absorbance of free LOF in the supernatant was measured by UV at 293nm and the amount of LOF was calculated according to above standard curve. The sedimentation was then lyophilized and weighted. The LC and EE of the nanoparticles were calculated as follows:

$$LC=(T-F)/M \quad (2)$$

Where T is total amount of LOF added into CS solution, F is free LOF amount in the supernatant, M is the total amount of nanoparticles.

$$EE=(T-F)/T \quad (3)$$

Where T and F are the same as the descriptions of equation (2).

2.7. In Vitro release behavior

0.01g nanoparticles were placed in a dialysis bag and put into a cell containing 100ml of pH 7.4 PBS solution under the condition of 37℃ and 100 rpm agitation. Subsequently series of 1ml dialysis solutions were withdrawn into 100 ml volumetric flask at specific time points (0.5 h, 1 h, 3 h, 5 h, 7 h, 10 h, 12 h, 24 h, 48 h, 72 h, 120h) and diluted. Then their absorbencies at 293nm were determined as above. The release dosage at each moment was calculated and drawn into cumulative release curve.

2.8. Statistics

Each experiment was performed triply and each data was expressed as an average.

3. Results and Discussion

3.1. Data analysis of orthogonal test

L_3^4 orthogonal experiments were designed. The four factors, such as CS concentration (A), TPP concentration (B), mass ratio of CS to LOF (C), mass ratio of CS to TPP (D), and their three levels were shown in Table 1.

In order to assess synthetically the effects of various factors on the combination properties of the nanoparticles, multiple index comprehensive weight analysis method was employed to deal with the data, where weighted coefficients of particle size (S_1), span (S_2), LC (S_3) and EE (S_4) were set as 0.2、0.1、0.3 and 0.4 respectively based on standard derivation and expert advice methodology.

The orthogonal experimental conditions and results were shown in Table 2. Since the region of optimality were referred to as a low particle size and span value, and a high LC and EE value. So the minimum value of either particle size or span among total column data was both set as 100 point, and similarly, the maximum value of LC or EE were set as 100 point. Then each result of size, span, LC and EE was changed into a certain point, and the combination score of each numbered experiment can be calculated referred to respective weighted coefficients, which was listed in the last column of Table 2.

Table 1 Factors and levels of orthogonal test

Levels	Factors			
	A Concentration of CS /mg/ml	B Concentration of TPP /mg/ml	C Mass ratio of CS to LOF	D Mass ratio of CS to TPP
1	1	1	0.5:1	3:1
2	1.5	1.5	1:1	4:1
3	2	2	2:1	5:1

The extreme difference of each factor (R_j value) was also listed in the last row of Table 2. These data indicate that the significance of four factors was in the order of A, C, D, B, which means concentration of CS, mass ratio of CS to LOF, mass ratio of CS to TPP affected greatly and concentration of TPP influenced slightly on the combination qualities of nanoparticles. In addition, by comparing the values of I_j, II_j, III_j, the significance of each factor can be concluded as following: $\mathrm{III}_A > \mathrm{II}_A > \mathrm{I}_A$, $\mathrm{III}_B > \mathrm{I}_B > \mathrm{II}_B$, $\mathrm{I}_C > \mathrm{III}_C > \mathrm{II}_C$, $\mathrm{II}_D > \mathrm{III}_D > \mathrm{I}_D$. Therefore $A_3B_3C_1D_2$, which corresponds to 2mg/ml CS concentration, 2mg/ml TPP concentration, 0.5:1 mass ratio of CS to LOF, and 4:1 mass ratio of CS to TPP, was regarded as the optimal combination parameters.

Table 2. Results of orthogonal experiments

NO.	Factors				Experiment results				Combination evaluations /points
	A	B	C	D	S1(nm)	S2	S3 (%)	S4 (%)	
1	1	1	1	1	249.0	2.37	5.32	16.24	39.80
2	1	2	2	2	157.4	0.99	3.73	15.27	35.67
3	1	3	3	3	112.7	0.77	2.14	19.36	38.66
4	2	1	2	3	276.7	4.72	5.95	21.08	47.73
5	2	2	3	1	272.2	4.97	4.27	26.55	51.12
6	2	3	1	2	251.5	1.77	8.56	27.53	79.90
7	3	1	3	2	779.2	1.73	7.31	34.17	72.35
8	3	2	1	3	410.0	1.19	7.83	25.53	68.59
9	3	3	2	1	310.2	6.07	6.44	22.74	49.98
I_j	114.1	159.9	188.3	140.9					
II_j	178.7	155.4	133.4	187.9					
III_j	190.9	168.5	162.1	155.0					
R_j	76.8	13.1	54.9	47.0					

3.2. Morphology of nanoparticles

LOF-loaded CS nanoparticles prepared under conditions of No.7 and No.8 in Table 1 were demonstrated in Fig.2. These nanoparticles were dispersed very well in suspensions with average diameters ranging from 410 to 779 nm. The TEM photos showed nanoparticles prepared under optimal condition had spherical biconcave in shape (Fig.3)., Their average diameter and span were 140nm and 0.95 respectively.

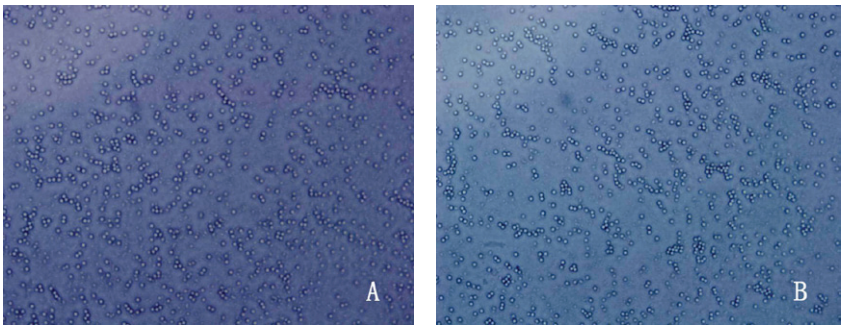


Fig.2. Photomicrographs of LOF-loaded CS nanoparticles (A) No.7 group (B) No.8 group

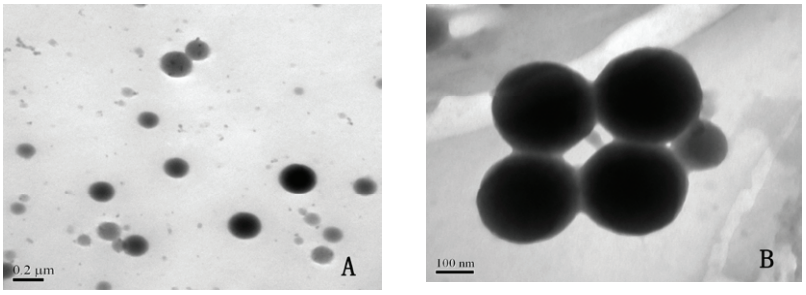
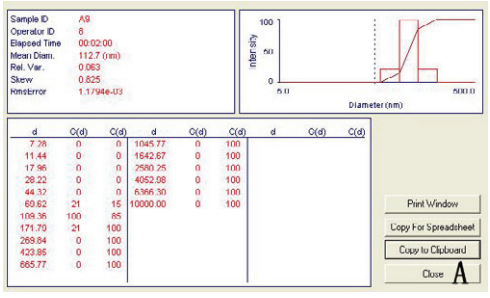


Fig.3. TEM of LOF-loaded CS nanoparticles

3.3. Particle size, LC and EE of nanoparticles

The size distributions of nanoparticles prepared under No.3 and No.7 were shown in Fig. 4, which demonstrated that the average diameters of No.3 and No.7 were 112.7nm and 779.2nm, and their spans were 0.77 and 1.73 respectively. Fig. 4(A) also showed that nanoparticles prepared under No.3 parameters had only one size of particles, whose diameters were from 69nm to 172nm. While the nanoparticles prepared under No.7 parameters had three compositions of particles, whose diameters were in the ranges of 1.0nm-1.8nm, 42nm-178nm and 562 nm-1778 nm respectively (Fig.4 (B)). The three groups of particles had huge differences between particle sizes, which determined the span value of No.7 was much greater than that of No.3. Perhaps the relative high concentration of CS caused the increased viscose of reactive solution, which resulted in not uniform particle formed.



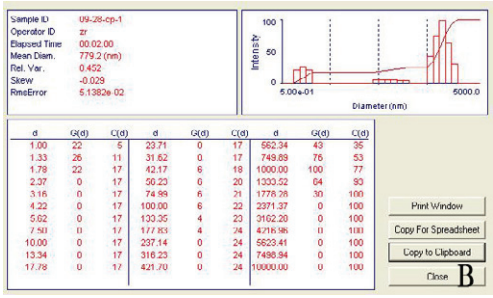


Fig. 4. Dynamic Light Scattering results of CS-LOF nanoparticles (A) No.3 group (B) No.7 group

The standard curve of LOF was shown in Fig. 5, where the scattered dots indicated the experimental data and the red line indicated the regressed equation:

$$A=0.0697C-0.0563 \ (r^2=0.9907) \quad (4)$$

Where A indicates absorbance of nanoparticle dissolution at 293nm; C indicates the LOF concentration and r^2 is related factors. The 0.9907 of r^2 value implies that the regressed equation matched well with the experimental data, and the equation can be used to assess the drug contents.

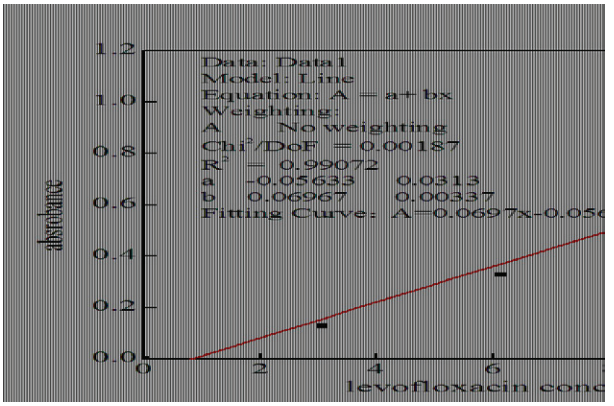


Fig.5. Standard curve of LOF

Based on Fig.5, LC and EE values of nanoparticles prepared under the optimal condition can be calculated as 6.13% and 24.91% respectively.

3.4. In vitro release behavior

The relationship between percentage of cumulative release and releasing time was demonstrated in Fig. 6. It can be observed that the initial release rate was relatively high with cumulative release percentage up to 10% within 0.5 h, 21.7% at 3h, 50% at 7.5h, 60% at 10h, 72% at 12h, while the subsequent release rates decreased gradually with about 85% at 24h, 90% at 56.5h. The initial higher release rates might be caused by the dissolution of surface drug from nanoparticles. At slight longer time, drug release might be mainly controlled by the diffusion process, which was much slower than the initial dissolution release. The drug release test indicated that the nanoparticles prepared under optimal condition could release LOF gradually up to three days.

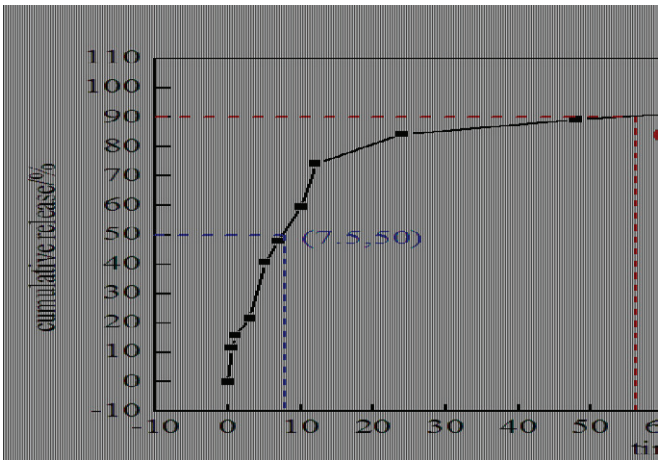


Fig.6. The curve of CS-LOF nanoparticles in vitro release

4. Conclusion

CS-LOF nanoparticles can be fabricated by ionotropic gelation method. The optimal preparation parameters were 2mg/ml CS concentration, 2mg/ml TPP concentration, 0.5:1 mass ratio of CS to LOF, and 4:1 mass ratio of CS to TPP. The optimal nanoparticles had spherical morphology, 140nm diameter, 0.95 span, 6.13% LC, and 24.91% EE. Thus prepared nanoparticles could release LOF gradually within 3 days. Antibacterial and biocompatible properties should be carried out in near future to evaluate its potential as sustainable delivery system.

Acknowledgements

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